Adh Locus in Maize for Detection of Mutagens in the Environment

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The advantages of the maize *Adh* system for low level mutagenesis studies are discussed. They include high mutation rate and the availability of both a strong selection system for null mutants and a sensitive method for detection of variants. The null selection involves treatment of pollen with allyl alcohol which is converted by the *Adh* enzyme to the toxic acrylaldehyde. *Adh* variants can be detected by alteration in affinity for phytic acid as well as thermal stability and electrophoretic mobility.

There are three factors which should be considered in selecting a genetic system for low frequency mutagenesis studies. A selective system for mutants is almost an absolute requirement when one is dealing with very low mutation rates. Secondly, it is desirable to study a sensitive gene which mutates at a higher than average rate. Thirdly, it would be advantageous if the gene specifies a protein in which changes in primary structure are readily detectable, changes in addition to those which lead to an alteration in such properties as enzymatic activity, net charge, and stability. The *Adh* (alcohol dehydrogenase) gene in maize fulfills all of these requirements and is ideally suited for studies on low frequency mutagenesis.

Adh is on the long arm of chromosome 1, about 1½ map units from *lw*. The ADH enzyme is a dimer (*1*). Both subunits of the dimer are enzymatically active and a dimer composed of one active and one inactive subunit has only one-half the specific activity of a dimer with two active subunits (*2*). In heterozygotes for alleles that specify active and inactive enzymes, the total enzymatic activity of the heterodimers is equal to that of the active homodimers although there are twice as many heterodimers as active homodimers. The dimer can be dissociated by freezing in the presence of a reducing agent and high salt, and can be readily re-associated with recovery of almost full activity (*3*).

The maize genome contains a second gene, *Adh2* (*4*); however, the presence of this gene does not interfere with the *Adh* analysis, since it specifies an enzyme with very low specific activity and is not active in pollen nor mature embryo except when the kernel is submerged in water for a prolonged period.

ADH is an inducible enzyme. Enzyme level in seedlings drops off rapidly with germination. After 5 days, very little activity can be detected in shoots or roots. However, enzymatic activity rises sharply when the seedlings are subjected to anaerobic stress (*5*). The rise in activity results from *de novo* synthesis of enzyme rather than activation of preexisting ADH protein (*6, 7*). It is of interest that the anaerobic treatment which induces the synthesis of ADH results in the cessation of most protein synthesis. ADH is one of only a relatively few major proteins synthesized under these conditions. Recent studies in my laboratory (*8*) have established that induction also involves *de novo* synthesis of ADH messenger RNA as determined by *in vitro* translation studies with RNA extracted from induced and uninduced seedlings.

The *Adh* gene is very sensitive to mutagenesis by ethyl methanesulfonate (EMS). The sensitivity of the gene to other mutagens was not tested since the purpose of our work was solely to obtain *Adh* mutants. EMS was the first mutagen tested and the results were so spectacular there was no need to try other mutagens.

The screen for the mutations of the *Adh* gene was conducted as follows: kernels homozygous for a

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particular *Adh* allele, as *Adh-F*, were soaked in a 0.08*M* solution of EMS at room temperature for 10 hr. After thorough rinsing with cold water the seeds were blotted dry and planted in the field. Plants grown from the treated seeds were used as female parents in crosses with plants homozygous for an *Adh* allele which specifies an electrophoretically distinguishable enzyme, as *Adh-S*. The ears were harvested and twelve kernels from scattered positions on each ear were analyzed electrophoretically for mutations of the *Adh-F* allele. Mutations resulting from EMS treatment of the seed occur as sectors of mutant kernels on the ear. Thus, although the kernel is sacrificed for the electrophoretic analysis, it is possible to recover the same mutation is other kernels from the ear sector. For the electrophoretic analyses, the kernels are simply soaked in water overnight, the scutella excised and squashed onto Whatman #3 filter paper squares which are inserted in the starch gel. After the run, the gels are specifically stained for ADH isozymes (*1*).

Mutations of *Adh-F* are recognized as deviations from the expected 1:2:1 ratio for the FF, FS and SS isozymes respectively. The untreated *Adh-S* allele acts as a built-in standard. Mutations at loci other than *Adh*, which could influence the synthesis of the ADH enzyme, are eliminated by the screen since they would affect both the *F* and the *S* alleles and not change the isozyme ratio. A number of mutant types have been recovered: (a) CRM – mutants which fail to produce a protein that can either dimerize with active protomers or react with anti-ADH antibodies; (b) CRM + mutants which specify enzymatically inactive protein; (c) mutants in which there is a decreased production of ADH protein (some of these may result from protomer instability); (d) mutant proteins which are stable in heterodimers but unstable as homodimers; (e) electrophoretic variants.

The frequency of mutations recovered at the *Adh* locus is very high. In three EMS mutagenesis experiments using three different alleles, we found that 3%, 1.5%, and 1.2% of the ears had a sector with a mutation at the *Adh* locus. The actual mutation rate is, of course, not that high since the ear develops from a few cells in the treated kernel. Also, the treated material is diploid so that there are at least two targets per cell and possibly four, if the chromosomes in the embryo of the mature kernel are in the G 2 stage of the cell cycle. It is very unlikely that any of the recovered mutants resulted from chromosome deletions. All showed normal transmission through pollen and egg but this does not rule out small deletions. Deletion of the *Adh* locus would, of course, result in a CRM-mutant. The strongest evidence against deletion as the basis for the CRM – mutations comes from the mutagnesis experiment with the *Adh-FCm* duplication (*9*). The duplicate *Adh* loci are very close, less than 0.01 map units apart, and have not as yet been separated by recombination. All of the 30 mutations recovered in this study involved either the *F* or the *Cm* locus and none were CRM-, as would be expected from deletions.

We have developed an effective selection procedure for ADH negative pollen grains. The technique should be useful in selecting mutations which occur at a low frequency. The enzyme specified by the *Adh* gene is present in mature pollen grains. It is synthesized during the gametophyte generation and results from the functioning of the *Adh* gene in the haploid nuclei of the gametophyte (*10*). This conclusion is based on the observation that pollen extracts from plants heterozygous for two *Adh* electrophoretic variants show only the two homodimer isozymes and no heterodimers. Heterodimers are formed, however, if the individual pollen grains carry two *Adh* alleles, as is the case with the *Adh-FCm* duplication. This is an important point, since is shows that synthesis of ADH enzyme in the pollen depends solely on the genotype of the pollen nuclei and is not influenced by the genotype of the diploid plant. The selection procedure for ADH negatives involves treatment of mature pollen with allyl alcohol. Allyl alcohol is a substrate for maize ADH and is oxidized to the highly toxic acrylaldehyde. Thus, pollen grains which contain active enzyme are killed and only the ADH negative pollen grains survive the treatment and function in fertilization to produce progeny kernels (*11*).

Any change in nucleotide sequence of a gene should be considered as a gene mutation. This should hold even for those base substitutions which do not alter the amino acid sequence of the specified protein such as substitutions in the third position of a number of codons. However, even many of the mutations which change the primary structure of a specified protein do not produce a mutant phenotype and thus are not detectable as mutations. Changes in electrophoretic mobility, enzymatic activity and stability are the criteria by which most mutant enzyme forms are generally recognized; however, for maize *Adh* and additional property of the enzyme serves to distinguish enzyme forms which do not differ in the properties listed above. The enzyme reversibly binds phytic acid (inositol hexaphosphate), a highly negatively charged molecule. Otherwise indistinguishable enzyme forms can differ in their affinity for phytic acid. As an example, we have tested eight EMS-induced electrophoretic variants which have identical charge, activity and stability. All eight differ in their affinity

for phytic acid as determined by the change in their electrophoretic mobility in the presence of this compound. The enzyme-phytic acid complex has a much higher net negative charge than that of the enzyme in the dissociated state and thus a more rapid electrophoretic mobility. The higher the affinity of the enzyme for phytic acid the more the equilibrium will be shifted toward the complexed state.

The *Adh* mutants which were recovered from the EMS treatment of the kernels described above, were unselected in that the progeny of all treated seed were tested for *Adh* mutations. Of the electrophoretic variants, all which showed a higher net negative charge differed from their progenitors by a single charge; however, quite unexpected results were obtained with the mutations which changed the net charge of the protein in the opposite direction. Of a total of 18 mutants with increased positive charge only two differed from their progenitors by a single charge while 16 differed by two charges. This preponderance of two charge change mutations was found with EMS treatment of alleles *Adh-S*, *Adh-F*, and *Adh-C* which specify enzymes of differing net charge. This result is unexpected since, according to the genetic code, the majority of single base substitutions in codons should lead to single charge changes. Single base substitutions in only two codons, GAA to AAA and GAG to AAG will cause a two-charge change by substituting the positively charged amino acid lysine for the negatively charged glutamic acid. The results could be explained by invoking hot spots or conformational differences in the mutants. According to the hot spot hypothesis, the guanine in a particular position in the *Adh* gene is very sensitive to EMS and is changed to an adenine at a higher than average frequency. This explanation has been ruled out since we have shown that the double-charge mutants derived from a common progenitor allele differ in their affinity for phytic acid. According to the hot spot hypothesis the mutants should be identical, since all would involve the same base substitution. We have also been able to rule out the conformer explanation. This hypothesis proposes that polypeptides can fold in either of two thermodynamically stable configurations depending on their primary structure. The net charge of the polypeptide depends on the number of charges which are exposed and the alternative conformational states differ by two in the number of exposed negative charges. Thus, any changes in amino acid

sequence which cause the polypeptide to fold into the alternative conformational state will appear as a two charge change. However, even this explanation is negated by the finding that the two charge difference between progenitor and mutant polypeptides is still observed in isoelectric focusing under denaturing condition in $9M$ urea. We are investigating the possibility that these mutants result from EMS-induced insertions of a transposable element into the *Adh* gene but to date they remain a mystery.

In conclusion, the *Adh* system is quite ideal for low level mutagenesis studies in that it is a highly mutable gene; the mutations are readily detectable by alterations in affinity for phytic acid in addition to changes in enzymatic activity, stability and electrophoretic mobility; and *Adh* negative mutations can readily be selected for in the pollen.

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